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<b>13. ABSTRACT (Maximum 200 Words)</b> Estrogens are one of the major agents responsible for the development of breast cancer disease. The effects of estrogens are mediated by estrogen receptor (ER) $\alpha$ and $\beta$ . The ER interacts with specific DNA sequences (EREs) of estrogen responsive genes and modulates gene transcription. The biochemical and physiological features of the newly discovered ER $\beta$ remains largely unknown. Estrogen responsive genes contain various imperfect EREs that deviate by one or more nucleotides from the consensus ERE. Since the nature of the ERE determines the transcriptional strength of ER $\alpha$ , differences in the binding affinity among EREs could also provide the receptors a way to display distinct gene induction properties. We measured the DNA binding affinity of ER $\beta$ to variant EREs derived from natural genes, using a gel mobility shift assay, and found that ER $\beta$ displays a DNA binding affinity and preference similar to that of ER $\alpha$ whether or not ligands are present. We further determined the contact sites of ERE utilized by the receptors using the missing nucleotide hydroxyl radical assay and found that the same nucleotides, particularly guanine residues in both strands, are critical for binding of both receptors. These results suggest that the nature of ERE is not a predictor for the receptor specificity.				
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## Introduction

Estrogen hormones trigger a broad array of physiological responses. They also play an important role in the etiology of breast cancer (1). Estrogen receptor (ER) alpha and beta are high affinity protein transcription factors that regulate expression of estrogen regulated genes (2) by binding to a *cis* acting element called estrogen responsive element (ERE). Most highly estrogen responsive genes contain multiple copies of estrogen responsive elements. Synergism is observed when gene is induced by two or more EREs which is greater than the sum of induction by individual ERE present alone (3, 4). Synergism contributes to the high level of transcription activation of ER alpha in transiently transfected cells. However, it still remains to be determined whether this phenomenon exists in natural genes within chromatin context. The newly identified ER beta is highly homologous to ER alpha in DNA binding domain. It is 30-60% homologous at other regions (5, 6). The physiological function of ER beta remains unclear. It was recently shown that ER beta acts as a transdominant inhibitor of ER alpha transcriptional activity (7), although studies with ER beta knock-out mouse (8) showed that the absence of ER beta had minimal effects on reproduction. Analyzing the DNA binding property and transcriptional responses including effectiveness and synergy would shed light on the mechanism of ER beta action.

## Body

### Generation of Stably Transfected Cells

The genomic DNA wraps around histone proteins and form chromatin structures. The chromatin structure plays an important role in the regulation of gene expression. Recent studies show that transiently transfected DNA is both structurally and functionally different from cellular chromatin (9). In order to determine whether ER alpha or ER beta can act synergistically when binding to multiple EREs in the chromatin context, we constructed stably transfected cells with ERE containing reporter genes integrated into genomic DNA.

To simplify the system, we first constructed the reporter gene driven by simple promoter and zero, one or two EREs (shown in Fig.1). The luciferase reporter cDNA and the promoter were inserted into pCI-neo mammalian expression vector (Promega, Madison, WI) containing selective marker. CHO cells transfected with the luciferase vectors were selected with antibiotic G-418. The integration of the luciferase reporter was screened through PCR on genomic DNA extracted from survival colonies.

A recent study (10) showed that the effectiveness with which ER alpha or ER beta activates transcription is strongly dependent on promoter and cell context. Presumably the function domains of each receptor respond differently to the protein activators unique to individual promoter. To investigate how ER alpha or ER beta induces transcription upon binding to multiple EREs within specific promoter context, we generated cell lines stably expressing luciferase reporter driven by the moderately strong thymidine kinase (TK) promoter. The

constructed reporter plasmids are shown in Fig.1. The promoter regions of estrogen responsive genes contain other elements in addition to ERE, such as AP-1 and SP-1. It was shown that ER alpha and ER beta can induce different transcriptional responses on AP-1 site upon binding to ligands (11). To investigate the responses of natural genes to ER alpha and ER beta in cells, we generated cell lines stably expressing luciferase reporter driven by pS2 or Cathepsin D gene promoters, which contain ERE and are estrogen responsive. The pS2 and Cathepsin D gene promoters are cloned through PCR from genomic DNA library and inserted into luciferase promoter region in the expression vector.

We transiently transfected ER alpha or ER beta expression vectors into various stably transfected cells described above in the presence of estradiol or 4-hydroxyltamoxifen and measured the expression levels of luciferase in response to ER. However, we could not detect any increase of luciferase expression in ER alpha or ER beta transfected cells comparing to those transfected with control vectors. The reason could be the low efficiency of transient transfection we used. The increase of luciferase expression in a small portion of cells is difficult to detect among the major untransfected cells. To solve this problem, we plan to deliver ER alpha or ER beta expression vectors into the stable cell lines by retrovirus system. The retrovirus system can transfect cells at almost 100% efficiency.

## The DNA Binding Property of ER beta *versus* ER alpha

### Gel Shift Analysis of ER alpha *versus* ER beta

Many reports analyzing estrogen-responsive genes revealed a minimal functional ERE consensus sequence, which is a 13-bp inverted repeat (13, 14, 15, 16). However, many estrogen responsive genes have been found to contain imperfect EREs, which vary from the consensus sequence by one or more nucleotides. One or more changes from the consensus sequence result in lower binding affinity of ER alpha to ERE (12, 17, 18). The different binding affinities of ER alpha to various EREs may modulate the estrogen response of individual gene depending on the sequence of ERE. In order to investigate the function of ER beta, we first characterized the DNA binding affinities of ER beta to various ERE comparing to ER alpha. We reasoned that if they have different binding preferences to various EREs, the two receptors may regulate different target genes.

We used natural EREs derived from gene promoters responsive to estrogen as depicted in Fig.2. Each substrate consists of a test sequence with one, two or three nucleotide changes (underlined) from the core consensus ERE embedded (box) within a larger oligomer with no ERE features called the "background oligomer". The DNA binding affinity of ER beta in comparison with ER alpha to these EREs is assessed by gel mobility shift assays. We used highly purified recombinant human ER alpha and ER beta from a baculovirus expression system obtained from a commercial source (PanVera, Madison, WI) and <sup>32</sup>P-end-labeled oligonucleotides. As shown in Fig.3, the amount of ER-bound DNA increases as the concentration of ER increases. At equimolar protein concentrations ER beta displays similar binding affinities to various EREs compared to ER alpha in the absence of ligand. A single change in the minimal inverted repeat ERE (13d1)

drastically reduces ER beta binding as previously reported for ER alpha (12). However, the binding affinities of ER beta to variant EREs with one or two changes are not significantly lower than those to consensus ERE when appropriate flanking sequences are present (15d1, 17d1 *versus* p15 and p17). The flanking sequences not only increase the affinity of ER beta to ERE but also rescue its binding to non-consensus. The results indicate that ER beta displays DNA binding affinity with a pattern and preference similar to that of ER alpha and suggest that the nature of and ERE-like sequence is not a predicament for the receptor specificity in the absence of ligand.

#### Gel Shift Analysis of ER Beta in the Presence of Ligands

ER functions by binding to ligands, undergoing conformational change, binding to ERE and interacting with coactivators. Ligand binding was shown to induce ER conformational change (19, 20). To test whether the DNA binding property of ER beta is altered by ligands, we investigated the binding affinities of ER beta to various EREs in the presence of 17 $\beta$ -estradiol, antiestrogen 4-hydroxyltamoxifen and ICI<sub>182,780</sub>. As shown in Fig.4, estrogen and antiestrogens do not alter the DNA binding affinities of ER beta and its binding preference.

#### Base Contacts of ER alpha *versus* ER beta on ERE sequences

Similar DNA binding properties of both ER receptors predict that both employ similar contact sites in an ERE. We tested this prediction by utilizing the missing nucleoside hydroxyl radical assay. This assay assesses the contribution to protein binding of each member of a base pair independently at all of the nucleotides in a linear double-stranded DNA molecule (21). This approach is based on the presumption that if a base important for binding is missing in a particular DNA molecule, the protein could not bind. Consensus (p17) and non-consensus (p17d2) EREs were treated with the hydroxyl radical to randomly remove single nucleotide from each DNA. Only one strand of DNA was labeled. ER beta or ER alpha was then allowed to bind the labeled, gapped ERE. The ER-bound DNA and free DNA were resolved by sequencing gel electrophoresis (shown in Fig.5A). A low intensity or missing band on the gel in the lane containing bound ERE, or conversely a high intensity band in the lane containing free ERE, identifies a nucleotide important for the formation of ER-ERE complex. The intensity of DNA bands was quantified by PhosphoImager. The ratios of free DNA to bound DNA at each base are plotted as shown in Fig.5B. A high ratio is observed at guanine in the half site TGACC at both strands for ER alpha-p17 interaction, suggesting that this guanine is critical for binding. The cytosine opposite to this guanine appears to be unimportant. The thymidine at position 1 and adenine at position 3 also show less intensity in the lane for bound ERE. The contact of ER alpha to the guanine at position 2 is consistent with other reports demonstrated by methylation interference experiments (22, 23). The crystal structure of ER alpha DNA binding domain (DBD) with ERE (24) also showed that the phosphate backbone adjacent to this G make multiple contacts to several amino acids in ER alpha. Truss et al. (22, 25) have shown the contact of the first T to ER alpha previously by potassium permanganate interference method. They did not observe the contact of the third A to ER alpha. However, crystal structure showed that this A is contacted by the protein (24). This is not surprising since the third position of the half site is the main difference between

ERE and glucocorticoid or progesteron responsive elements (GRE/PRE) and is essential for discrimination between the two classes of responsive elements. Higher ratios of free to bound DNA at the same bases have been observed for ER beta-p17 complex formation. The overall plot pattern of ER beta-p17 interaction is similar to that of ER alpha-p17 (Fig.5B), suggesting that both receptors make similar contacts to consensus ERE. When two nucleotides were mutated (p17d2), some changes of contact sites were introduced at the lower strand. The T to A change at the first position of the half site (T(/A)GA(/C)CC) did not have significant effect. The second G remained to be the most important nucleotide for interaction. The contact of the third position to protein was lost by mutation, instead, the cytosine at the fifth position became more important, as well as its adjacent bases. The change of nucleotides resulted in new formation of hydrogen bond networks between ER DBD and ERE. ER beta was shown to have similar contact sites on non-consensus ERE. These results confirmed our hypothesis that both receptors interact with ERE in a similar manner.

### The Localization of ER beta in Cells

See appended manuscript.

### Key Research Accomplishments

- Construction of estrogen responsive reporter plasmids containing various promoters including multiple EREs embedded in simple or moderately strong promoters and natural gene promoters
- Generation of cell lines with reporter gene and its promoter integrated into genome
- Test of the DNA binding affinities of ER alpha and ER beta to various consensus and non-consensus ERE in the absence and presence of estrogen and antiestrogens
- Identification of the contact sites of consensus and non-consensus ERE by ER alpha and ER beta
- Determination of the localization of ER beta in transfected cells in the absence and presence of various ligands

## Reportable Outcomes

### Manuscripts:

Sathya, G., Yi, P., Raubertas, R. F., Hilf, R., Bambara, R. A. Muyan M, (2000) Role of Estrogen Receptor  $\alpha$  Activation Domains in Estrogen-induced Synergistic Response. *To be Submitted.*

Muyan, M., Yi, P., Sathya, G., Willmert, L., Driscoll, M. D., Hilf R, Bambara, R. A. (2000) Fusion Estrogen Receptors as Models for Analysis of the Functional Properties of Dimeric Receptors. *Mol. Endo., Submitted.*

### Abstracts:

Yi, P., Muyan, M., Driscoll, M.D., Sathya, G., Hilf, R., Bambara, R.A. (1999) Characterization of the Biochemical and Functional Properties of Estrogen Receptor beta  
*4<sup>th</sup> Annual Scientific Symposium, University of Rochester Cancer Center* p.73.

Muyan, M., Yi, P., Hilf, R., Bambara, R.A. (2000) A Fusion Estrogen Receptor Variant Generated by Genetic Conjugation Displays an Effective Dominant Negative Phenotype. *The Program and Abstracts of the 82<sup>nd</sup> Annual Meeting of the Endocrine Society*, p.190: 771.

Yi, P., Bhagt, S., Hilf, R., Bambara, R.A. Muyan, M. (2000) Does the Binding Affinity of Estrogen Receptor beta to Various Estrogen Responsive Elements Correlate with Transcriptional Strength of the Receptor? *The Program and Abstracts of the 82<sup>nd</sup> Annual Meeting of the Endocrine Society*, p. 359: 1493.

### Poster Presentations:

University of Rochester Cancer Center, 4<sup>th</sup> Annual Scientific Symposium, Rochester, New York, September, 1999

Department of Defense Breast Cancer Research Program Meeting, Atlanta, Georgia, June, 2000

The Endocrine Society's 82<sup>nd</sup> Annual Meeting, Toronto, Canada, June, 2000

### Degree obtained:

Ganesan Sathya	Ph.D., 2000
Ping Yi	M.S., 2000

### Employment or Research Opportunities Received:

Ganesan Sathya	Post-doctoral position in Duke University Medical Center, Pharmacology and Cancer Biology
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## Conclusions

We constructed reporter luciferase plasmids driven by simple and moderately strong promoters with zero, one or two EREs embedded in and the reporters driven by natural estrogen responsive gene promoters. We stably transfected these plasmids into ER negative cell line – CHO cells and successfully select colonies that have the integration of the reporters into genome. These cell lines will be important for revealing the physiological responses of ER alpha and beta within chromatin context and the gene responses induced by estrogen and antiestrogens.

We tested the DNA binding affinities of ER beta to various consensus and non-consensus EREs with or without appropriate flanking sequences. The flanking sequences increase the binding affinity of ER beta and tolerate one or two nucleotide changes. The binding affinities of ER beta to EREs are similar to those of ER alpha and binding to estrogen and antiestrogens does not alter them.

We further determined the contact sites of ERE to both ER alpha and beta by utilizing missing nucleoside hydroxyl radical assay. We found that the two receptors contact the consensus and non-consensus EREs in a similar manner. The results suggest that the DNA binding properties of ER beta are similar to those of ER alpha.

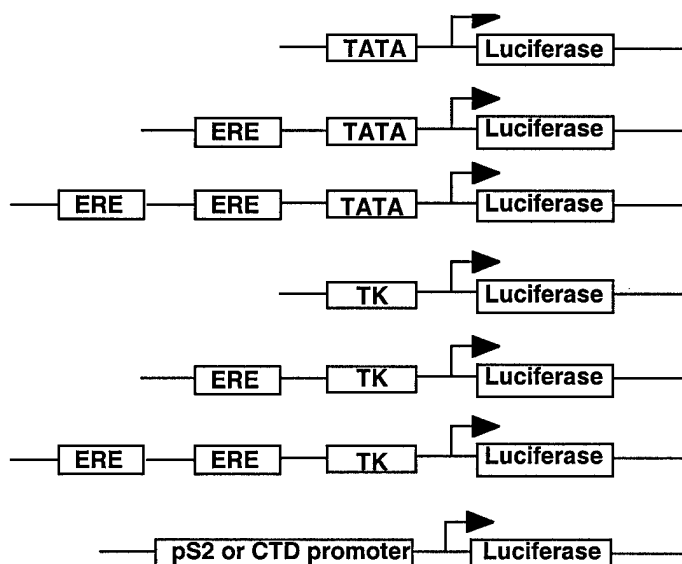
However, the transcriptional effectiveness of the two receptors is dramatically different. The transcriptional responses seem not relate to DNA binding. DNA was shown to be an allosteric effector for ER. Both ligand and DNA can induce the conformational change of proteins, which, in term, determines the interaction of receptors to coactivators. This interaction is critical for the transcription responses induced by ER. To further investigate the mechanism of ER action, we will assay ER conformation upon binding to ligands and ERE by protease digestion. We will also investigate the interaction of coactivators to ER alpha and beta under these conditions.

We have investigated the localization of ER beta in transient transfected cells in the absence and presence of ligands using immunocytochemistry. The results showed that ER beta is constitutively localized in nucleus similar to ER alpha regardless the poor homologous to the nuclear localization region of ER alpha.

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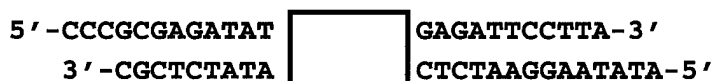
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## Figures



**Fig.1** Constructed plasmids to test the synergy of ER upon binding to multiple EREs within chromatin context and under control of different promoters. TK: thymidine kinase promoter, moderately strong promoter. pS2 or CTD (Cathepsin D): natural estrogen responsive gene containing ERE in the promoter region.

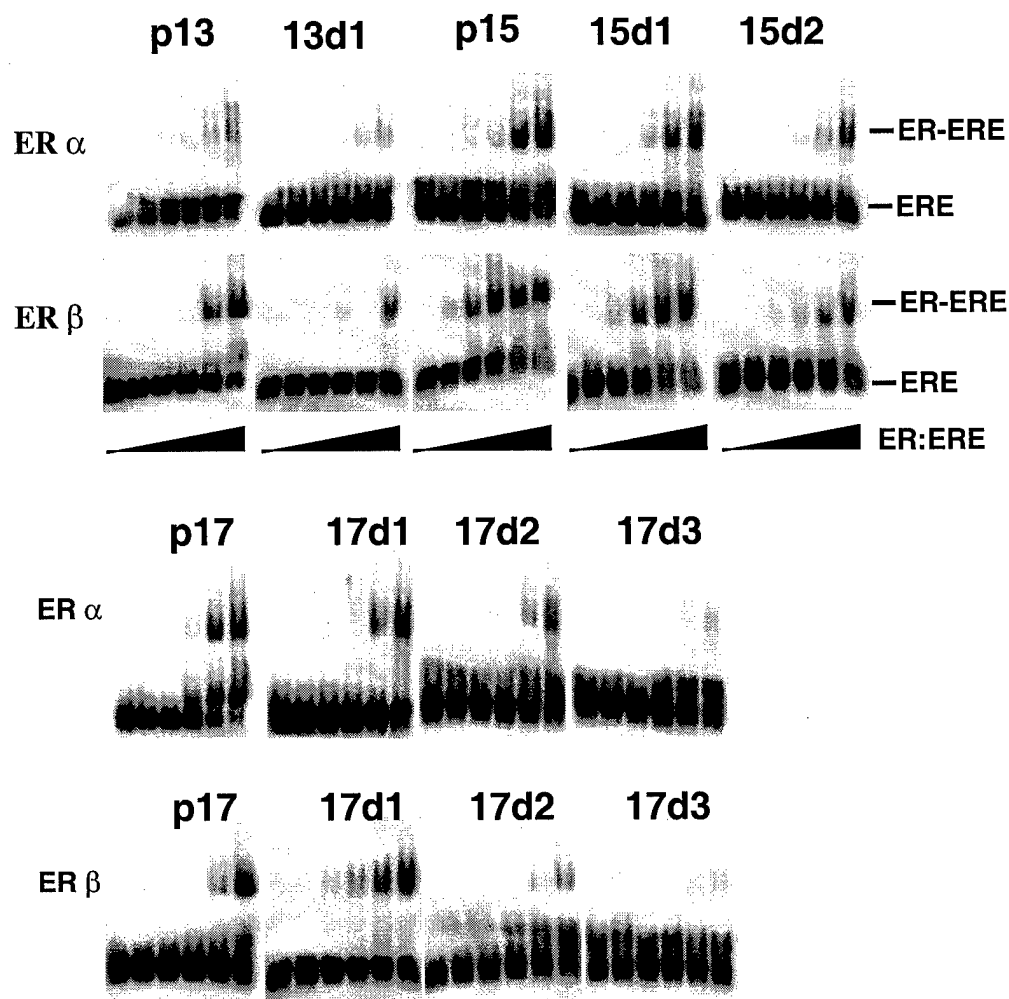
### Background Oligomer



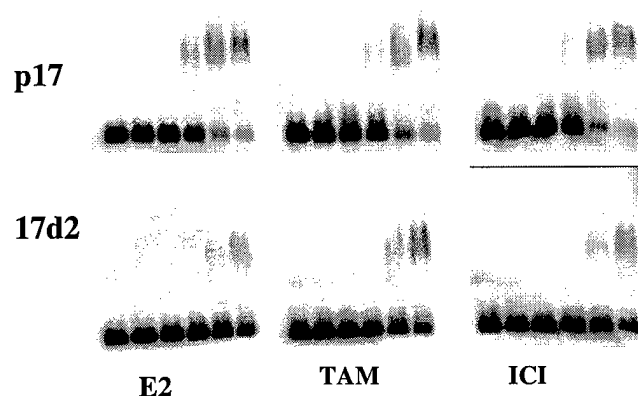
### Test Sequence

GGTCAcattGACC	p13
GGGCAcattGACC	13d1
AGGTCAcattGACCT	p15
AGGGCAcatTGACCT	15d1
AGGGCTcatTGACCT	15d2
CAGGTCAcattGACCTG	p17
CAGGGCAcatTGACCTG	17d1
CAGGGCTcatTGACCTG	17d2
CAGCGCTcatTGACCTG	17d3

**Fig.2** Test ERE sequences.

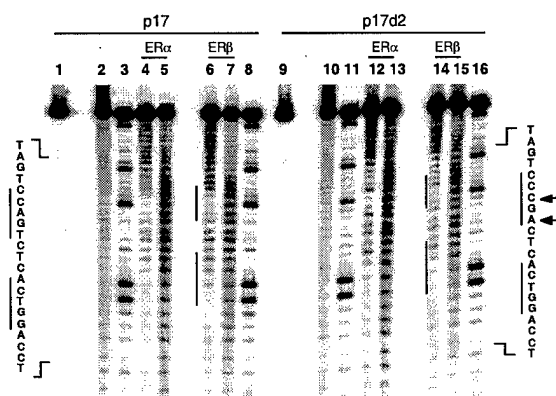


**Fig.3** Binding of ERs to various ERE sequences as analyzed by gel mobility shift assay.

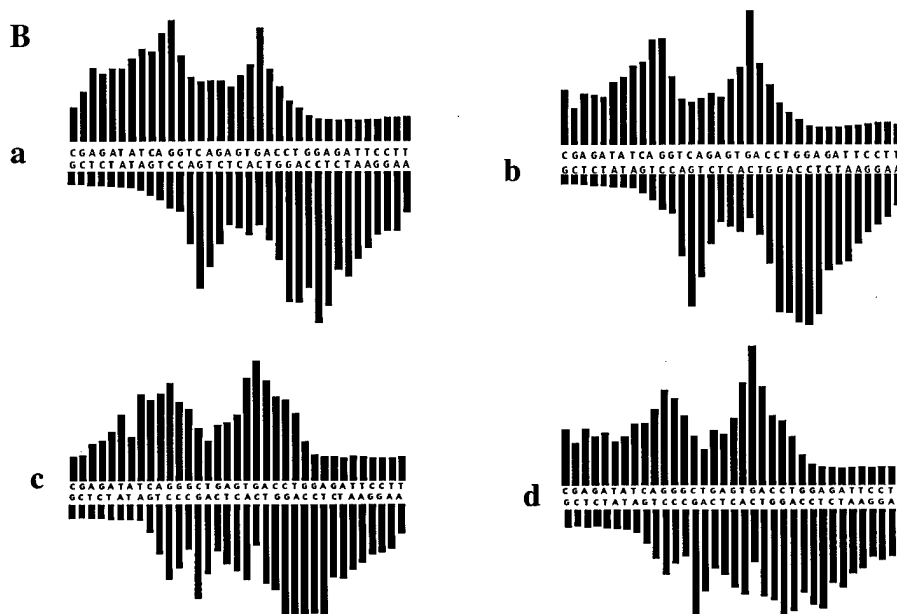


**Fig.4** ER ligands do not alter the DNA binding affinities of ER  $\beta$  to consensus (p17) and non-consensus (17d2) EREs as analyzed by gel mobility shift assay. E2:17 $\beta$ -estradiol, TAM: 4-hydroxyl tamoxifen, ICI: antiestrogen ICI182,780.

A



B



**Fig.5** ER $\alpha$  and ER $\beta$  have the same contact sites on perfect and imperfect EREs, as determined by the missing nucleoside approach. **A.** The DNA was digested by hydroxyl radical under condition by which at most one cut per DNA molecule occurs. The ER bound digested DNA and free DNA were separated on native gel. The DNA was isolated and resolved by a sequencing gel. Lane 1-8, the oligonucleotide containing p17 perfect ERE. Lane 9-16, p17d2 imperfect ERE. Lanes 4, 6, 12 and 14 are ER bound DNA. Lanes 5, 7, 13 and 15 are free DNA. Lane 1 and 9 are control DNA without digestion. Lane 2 and 10 are digested DNA without any protein binding. Lane 3, 8, 11 and 16 are specifically digested at G nucleotide to show the position of each nucleotide. The vertical lines indicate the position of ERE. The sequences of the EREs are shown. The arrows indicate the mutated nucleotides in p17d2 sequence. **B.** The intensity of ER bound and free DNA lanes at each nucleotide band as described in panel A were quantified and analyzed by PhosphorImager. The ratio of free DNA to bound DNA is shown. A high ratio indicates the nucleotide is important for ER binding to ERE. **a.** ER $\alpha$  binds to p17. **b.** ER $\beta$  binds to p17. **c.** ER $\alpha$  binds to p17d2. **d.** ER $\beta$  binds to p17d2.

# **NUCLEAR LOCALIZATION OF HUMAN ESTROGEN RECEPTOR (ER) $\beta$ IS INDEPENDENT FROM ER-LIGANDS IN TRANSFECTED MAMMALIAN CELLS**

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**Key words:** estrogen receptor, immunocytochemistry, intracellular localization, ER-ligands, transfected mammalian cells

## ABSTRACT:

The synthesis of ER $\beta$  in various human tissues implies that this ER subtype plays a major role in both physiology and pathophysiology of estrogen signaling. Although primarily localized in the cell nucleus of diverse human tissues, an ER $\beta$ -like immunoreactivity is also detected in the cytoplasm of various cells of the reproductive system. The role of estrogens and antiestrogens in the intracellular distribution of ER $\beta$  is yet unknown. We examined whether the intracellular distribution of ER $\beta$  is affected by ER-ligands in transfected mammalian cells using an immunocytochemistry approach and show here that ER $\beta$ , like ER $\alpha$ , is constitutively localized in the nucleus.

## INTRODUCTION:

Estrogens influence the differentiation, development and function of organs of the reproductive system and mammary gland. They are also important regulators of bone and cardiovascular system homeostasis. Until recently, estrogen receptor (ER)  $\alpha$ , a member of the steroid/thyroid hormone receptor superfamily that acts as a hormone-inducible transcription factor, was thought to be the sole mediator of these diverse actions of estrogens. The identification of the novel ER $\beta$  encoded by a different gene (1, 2) has led to re-evaluation of the physiology and pathophysiology of estrogen signaling. Reverse transcriptase-polymerase chain reaction, RNase protection assays and *in situ* hybridization techniques have provided evidence that the expression of the ER $\beta$  and ER $\alpha$  genes displays overlapping and distinct tissue distributions (3, 4). Although ER $\alpha$  and ER $\beta$  share high amino-acid sequence homology and display similar biochemical and functional properties (5), they also possess different structural characteristics responsible for differential transcriptional activation of estrogen target genes. Studies elucidating the molecular mechanism of action of ER $\beta$  using heterologous expression systems indicated that the response of reporter genes to this receptor can be distinct from that observed for ER $\alpha$  and is dependent on cell- and promoter-context (6, 7). These imply that the ER subtypes can regulate estrogen signaling convergently and divergently dependent upon tissue of expression.

Selective intracellular compartmentalization of the steroid receptors is thought to be one mechanism by which transcription of target genes are modulated (8). While ER $\alpha$  and progesterone receptors are localized constitutively in the nuclei of both native tissues of origin and transfected cells, unliganded glucocorticoid, mineralocorticoid and androgen receptors reside in the cytoplasm from where ligand binding induces translocation of the receptors to the nucleus. Recent immunohistochemical studies provided evidence that an ER $\beta$ -like immunoreactivity is observed in the nuclei of various tissues from human, intact and gonadectomized animals (9-12). In human, an ER $\beta$ -like immunoreactivity is also detected in the cytoplasm of ovarian corpus luteal cells, endometrial luminal epithelia, epithelial layer of the cervix and the prostate, while ER $\alpha$  is absent or predominantly localized in the cell nucleus of these tissues (10). A cytoplasmic staining for ER $\beta$  was also reported in some cells of the gonadectomized rat forebrain (12). These results suggest potential differences in the intracellular distribution of ER $\beta$  depending on the tissue of expression, endocrine status and sex of donor species.

The hinge region, or "D" domain, of ER $\alpha$  contains multiple proto-signals that regulate cooperatively the nuclear targeting of the receptor (13). One of the least conserved regions in ER $\beta$  compared to ER $\alpha$  is the hinge domain, having about 30% homology. Hence, the unique structural characteristics of the hinge region of ER $\beta$

may lead to the differential intracellular distribution of the receptor depending upon the presence of ER-ligands. We addressed this issue using gene transfer techniques into heterologous ER-negative cells. This approach provides a controlled environment to study and compare the intracellular localization of ERs in response to ER-ligands in the same cell type by immunocytochemistry. This method also avoids tissue specific variations, and sex and gonadal status. We report here that ER $\beta$ , like ER $\alpha$ , is localized in the nuclei of the transfected mammalian cells whether or not cells were treated with 17 $\beta$ -estradiol (E<sub>2</sub>), partial agonist 4-hydroxy tamoxifen (4-OHT), an active metabolite of the widely used antiestrogenic compound tamoxifen, or pure antagonist ICI 182,780 (ICI).

## MATERIALS AND METHODS

### *Plasmids*

The human wild-type ER $\alpha$  cDNA from an expression plasmid (provided by the late Dr. Angelo Notides, University of Rochester, Rochester, NY) was inserted into a mammalian expression vector pM<sup>2</sup>-AH (provided by Dr. Irving Boime, Washington University, St. Louis, MO) digested with *Sal I* and *BamH I* enzymes. This expression vector contains the Harvey Sarcoma Virus Long Terminal Repeat as the promoter. The human ER $\beta$  cDNA in an expression vector was kindly provided by Dr. Simak Ali, Imperial College of Medicine, London, United Kingdom. This cDNA encodes a 477 amino acid long ER $\beta$ . The extended sequence encoding the additional amino-terminal 53 amino acids was generated using PCR from human placental DNA (Sigma-Aldrich, St. Louis, MO) with primers based on the published sequence of the full length ER $\beta$  (14). Following amplification, PCR product was digested with *Sal I* and *Msc I* and inserted into the vector bearing the parent "short" ER $\beta$  cDNA linearized with the same enzymes. The resultant "long" ER $\beta$ , referred here as the wild-type ER $\beta$ , was sequenced. In construction of the ER $\alpha$  and ER $\beta$  with an amino terminal Flag epitope, an *Nde I* restriction enzyme site was introduced at the first codon of both the ER $\alpha$  and ER $\beta$  cDNA using PCR. A DNA fragment bearing 5' *Sal I* and 3' *Nde I* restriction enzyme sites was constructed using oligonucleotide pairs synthesized by Sigma-Genosys Inc., The Woodlands, TX. The oligonucleotides were annealed and inserted into the pBluescript II KS (+) bearing either the ER $\alpha$  or the ER $\beta$  cDNA and linearized with the same restriction enzymes. This DNA fragment contains a Kozak sequence followed by an ATG sequence as the first codon encoding methionine. This was followed by in-frame sequences that encode the eight amino acid long Flag epitope. This epitope was sequenced to ensure the correct sequence. Synthesis of constructs was assessed by translation *in vitro* using a kit (Promega) followed by Western blotting using HC-20 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), PA1-313 (Affinity BioReagents, Golden, CO) or M2 (Sigma-Aldrich) antibody. The ER $\alpha$  and ER $\beta$  cDNAs with or without Flag epitope were synthesized as proteins with an estimated M<sub>r</sub> 67 and 60 kDa, respectively (data not shown). The truncated ER $\alpha$  variant without or with amino-terminal Flag epitope contains only the carboxyl terminus E and F domains of the receptor and was constructed by PCR. The introduced stop codon (TAA) at the 3' end is within the context of polyadenylation signal (TAATAAA). The construct was synthesized *in vitro* to a protein with an estimated M<sub>r</sub> 35 kDa as assessed by western blotting using HC-20 or M2 antibody.

### *Cell culture, Transfection and Immunocytochemistry*



COS-1 and CHO cells were maintained in DMEM and F-12 K media (Washington University Tissue Culture Center, St. Louis, MO), respectively, containing 10% bovine fetal serum (HyClone, Logan, UT) with 0.5% penicillin/streptomycin mixture (Gibco-BRL, Grand Island, NY). Cells (50,000/well) were plated onto cover glasses (18 mm circle, VWR Scientific Inc., PA) in 12-well tissue culture plates containing media (without phenol red) supplemented with 5% charcoal-dextran treated fetal bovine serum (HyClone) and 0.5% penicillin/streptomycin mixture. After 48 hr, cells were transiently transfected with 1  $\mu$ g DNA using 5  $\mu$ l of Superfect transfection reagent (Qiagen, Santa Clarita, CA) as recommended by the manufacturer and incubated for 3 hr at 37°C. Cells were then washed twice with phosphate-buffered saline (PBS) and further incubated in media in the absence or presence of 10<sup>-9</sup> M 17 $\beta$ -estradiol (E<sub>2</sub>, Sigma-Aldrich), 4-hydroxytamoxifen (4-OHT, Sigma-Aldrich) or ICI 182,780 (ICI, Tocris, Ballwin, MO) for 24 hr.

DNA for transfection was prepared using the Qiagen Endotoxin Free Maxi Prep Kit.

For immunocytochemistry, cells were fixed in 2% paraformaldehyde in PBS for 30 min, permeabilized with 0.4% Triton X-100 in PBS for 10 min. Following an extensive wash, cells were incubated with 10% normal goat serum (Sigma-Aldrich) in PBS for 1 hr. This was followed by incubation with a primary antibody (1:100 dilution) in PBS containing 2% normal goat serum (Sigma-Aldrich) for 2 hr. The primary antibodies were HC-20, PA1-313 and M2. Cells were washed and further incubated in fluorescein conjugated (FITC, Santa Cruz Biotechnology) secondary antibody (1:200 dilution) in PBS containing 2% normal goat serum for 30 min. The cover glasses were mounted on a glass slide (VWR Scientific) using a mounting media containing DAPI (Vectashield, Vector Laboratories, Burlingame, CA) for nucleus staining and examined by fluorescence microscopy.

Subcellular localizations of the ERs in transfected mammalian cells were independent of the expression vector (pM<sup>2</sup> *versus* pcDNA3.1; Invitrogen, Carlsbad, CA) bearing the receptor cDNA, the cell staining procedure (immunofluorescence *versus* immunoperoxidase) employed or the dilution of the first antibody (1:50 *versus* 1:100). All experiments were repeated at least two different times in duplicate.

## RESULTS AND DISCUSSION:

Immunohistochemical studies in humans and animals suggest that an ER $\beta$ -like immunoreactivity can be detected in the nucleus and also cytoplasm depending on the tissue of expression (9-12). The role of estrogens in the intracellular distribution of ER $\beta$  is yet unknown. The hinge region of ER $\alpha$  contains multiple signals that regulate cooperatively the nuclear targeting of the receptor (13). One of the least conserved regions in ER $\beta$  compared to ER $\alpha$  is the hinge domain (1, 2). Thus, the unique structural characteristics of the hinge region of ER $\beta$  may lead to the differential intracellular distribution of the receptor depending upon the tissue of expression and presence of ER-ligands. We therefore examined whether the intracellular compartmentalization of the ER $\beta$  is altered by the presence of ER-ligands. To address this issue, we transiently transfected ER negative COS-1 cells with the mammalian expression vector pM<sup>2</sup> bearing ER $\beta$  cDNA, or no cDNA as a control. We then assessed the intracellular localization of the receptor by immunocytochemistry, using an antibody specific to the carboxyl-terminus (PA1-313). We also transfected cells with pM<sup>2</sup> bearing ER $\alpha$  cDNA for comparative analysis. We detected intracellular localization of the receptor by an antibody specific to the carboxyl-terminus (HC-20). We observed no intracellular staining with either the ER $\beta$  or the ER $\alpha$  antibody in cells transfected with the expression vector lacking cDNA (data not shown); whereas, the ER $\beta$  or the ER $\alpha$  was

predominantly localized in the nucleus in the absence of ER-ligand (Fig. 1A). To ensure that the nuclear localization of the ER subtypes is not observed only with the antibody specific to the carboxyl-terminal epitope of the receptors, variant ER cDNAs bearing sequences that encode an amino-terminal Flag epitope were transfected into COS-1 cells. The Flag (M2) or the receptor-specific (data not shown) antibody detected both ER $\beta$  and ER $\alpha$  localized predominantly in the nuclei of cells. These results confirm the nuclear localization of both ERs in COS-1 cells in the absence of ER-ligand. Staining confined to nuclei with both the receptor-specific and Flag antibodies shows that the amino terminal Flag epitope did not alter the intracellular compartmentalization of the receptor.

Evidence that predominant nuclear localization depends on the structural integrity of the receptor proteins was obtained by transfection of the expression vector bearing a truncated ER $\alpha$  variant ( $\alpha$ EF) cDNA that contains only the carboxyl-terminal E/F domain sequences into COS-1 cells. The variant receptor bears an amino-terminal Flag epitope and lacks the entire amino-terminal A/B region along with the DNA binding and hinge domains. Due to the size of the protein and the lack of hinge domain, this construct should show a diffuse intracellular staining encompassing both the nucleus and cytoplasm (13). Indeed, this is what we observed; the truncated receptor was localized in both the nucleus and cytoplasm detected with the receptor-specific HC-20 or M2 antibody (Fig. 1C). This distribution was independent of ER-ligand (data not shown).

Studies have indicated that E<sub>2</sub>, 4-OHT and ICI bind to ER $\alpha$  and ER $\beta$  with high affinity (inhibition constant, K<sub>i</sub>, ranging from 0.04 to 0.4 nM) (5). The treatment of cells with a saturating concentration (1 nM) of E<sub>2</sub>, 4-OHT or ICI for 24h had no effect on the intracellular distribution of either ER subtype (Fig. 1D-F). These results demonstrate that ER $\beta$ , as ER $\alpha$ , is constitutively localized in the nuclei of the transfected COS-1 cells.

We also transfected ER negative CHO cells with expression vectors bearing the Flag-ER $\beta$  or the Flag-ER $\alpha$  cDNA to ensure that the nuclear localization of ERs is also not cell-specific. As observed in COS-1 cells, both ER $\beta$  and ER $\alpha$  were detected in the nuclei of CHO cells using antibodies specific for either receptors (Fig. 1G) or the Flag epitope (Fig. 1H). Treatment of cells with 1 nM E<sub>2</sub>, 4-OHT or ICI for 24h did not alter the intracellular localization of the ER subtypes (data not shown). These findings further demonstrate that ER $\beta$ , as ER $\alpha$ , is primarily localized in the nuclei of the transfected mammalian cells independent of ER-ligand.

The predominant nuclear localization of ER $\beta$  in transfected cells is consistent with immunohistochemical observations in which an ER $\beta$ -like immunoreactivity was detected in the nuclei of cells of various tissues from human and animals (9-12). However, Taylor and Al-Azzawi (10) also detected ER $\beta$  in the cytoplasm of some cells. The presence of cytoplasmic ER $\beta$  was particularly evident in tissues derived from the human reproductive system, while ER $\alpha$  was either absent or predominantly nuclear. Although the underlying reason is not clear, several possibilities need to be considered. 1) The sensitivity of antibodies we used could have been below the threshold needed to detect low levels of the receptor present in the cytoplasm in transfected mammalian cells by immunocytochemistry. This seems unlikely, because one of our antibodies (PA1-313) is the same as that used by Taylor and Al-Azzawi (10) in their immunohistochemistry approach. Moreover, truncated ER $\alpha$  variant (F- $\alpha$ EF) containing only the carboxyl-terminal E and F domains showed a diffuse intracellular staining encompassing both the nucleus and cytoplasm using either the Flag or the receptor specific antibody. This indicates that both antibodies are able to detect the receptor variant irrespective of its localization within the cell. 2) The immunohistochemistry studies were done using human tissue samples

obtained from adult human cadavers or from patients at the time of surgery for various pathological conditions, all with unknown reproductive status (10). Thus, differences in experimental conditions together with use of immunohistochemistry *versus* immunocytochemistry approaches could have led to differences in the intracellular localization of ER $\beta$ . 3) The expression of both ER subtype genes was shown to fluctuate with the menstrual cycle (15), presumably level of receptor synthesis. These altered levels of ER $\beta$  in reproductive tissues *versus* transiently transfected cells could have affected its intracellular distribution, producing differences in the intracellular compartmentalization of the receptor. However, we utilized a heterologous expression system that over-synthesizes the protein of interest. We detected no cytoplasmic staining for both receptor subtypes, whether or not ER-ligand was present, by using antibodies specific to either the carboxyl-termini of the receptors or the amino-terminal Flag epitope. This implies that the extent of *de novo* synthesis of receptor proteins is unlikely to be responsible for the differences in the intracellular localization of the ER $\beta$ . 4) Transcripts encoding ER $\beta$  variant proteins with altered structural features have been detected in human tissues including the reproductive system (16-19). These ER $\beta$  variants could selectively localize in the cytoplasm depending on the tissue of expression and the endocrine status of donors. This could have led to immunohistochemical detection of ER $\beta$  species in the cytoplasm as well, similar to our observation for the truncated ER variant that localized in both the nucleus and cytoplasm.

We, nevertheless, show here that ER $\beta$ , like ER $\alpha$ , is predominantly localized in the nuclei of transfected mammalian cells independently from ER-ligands.

The transport of nuclear proteins into the nucleus is a selective process and occurs through protein-specific nuclear localization signals (NLSs) (20). NLSs are short stretches of amino acids thought to interact with proteins in the cytoplasm, on the nuclear envelope and/or at the nuclear pore complex (20). The hinge domain of ER $\alpha$  has been shown to possess three NLS stretches that function cooperatively for an effective transport of the receptor into the nucleus (13). The hinge regions of human ER $\beta$  and ER $\alpha$  have poor homology (14). The structural analysis of the hinge domain of ER $\beta$  coupled with immunocytochemistry in heterologous cell systems would help to examine whether this ER subtype contains a similar protein-specific NLS.

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# FIGURE LEGEND:

**Figure 1.** Intracellular localization of human estrogen receptor (ER)  $\beta$  and  $\alpha$  in transfected mammalian cells. COS-1 (A-F) and CHO (G and H) cells were transiently transfected with a mammalian expression vector bearing cDNA for either ER $\beta$  or ER $\alpha$  without ( $\beta$  and  $\alpha$ ) or with Flag epitope (F- $\beta$  or F- $\alpha$ ). COS-1 cells were also transiently transfected with a truncated ER $\alpha$  variant (F- $\alpha$ EF). This variant contains only the carboxyl-terminal E and F domains and bears an amino-terminal Flag epitope (C). Following transfection, the cells were treated without (A-C and G, H) or with 1 nM estradiol 17- $\beta$  ( $E_2$ ) (D), 4-hydroxytamoxifen (4-OHT) (E) or ICI 182,780 (ICI) (F) for 24 hr. The cells were then fixed, permeabilized and probed with antibodies specific for ER $\alpha$  (HC-20), ER $\beta$  (PA1-313) or Flag epitope (M2). The receptor proteins were visualized with a fluorescein-conjugated secondary antibody (FITC). DAPI staining indicates the nucleus.

